GEORGIA INSTITUTE OF TECHNOLOGY
OFFICE OF CONTRACT ADMINISTRATION
SPONSORED PROJECT INITIATION

Date: 1/12/80

Project Title: Enzymatic Epoxidation and Oxygen Activation

Project No: G-33-655

Project Director: Sheldon May

Sponsor: NSF

Agreement Period: From 12/15/79 Until 11/30/83 ~~5/31/82~~ 83

Type Agreement: Grant No. PCM-7918334

Amount: $70,000 G-33-655
2,427 G-33-353 (Cost Sharing)
$72,427 TOTAL

Reports Required: Annual Progress Report (w/request for renewal); Final Project Report

Sponsor Contact Person (s):

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Defense Priority Rating: N/A

Assigned to: Chemistry (School/Laboratory)

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SPONSORED PROJECT TERMINATION/CLOSEOUT SHEET

Date 4/23/85

Project No. G-33-655 School/~~Lab~~ XXXX Chem

Includes Subproject No.(s) _____

Project Director(s) Sheldon May GTRC ~~GIT~~ XXXX

Sponsor NSF

Title Enzymatic Epoxidation and Oxygen Activation

Effective Completion Date: 11/30/83 (Performance) 2/29/84 (Reports)

Grant/Contract Closeout Actions Remaining:

- [ ] None
- [ ] Final Invoice or Final Fiscal Report
- [ ] Closing Documents
- [X] Final Report of Inventions For our records.
- [ ] Govt. Property Inventory & Related Certificate
- [ ] Classified Material Certificate
- [ ] Other _____

Continues Project No. _____ Continued by Project No. _____

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FORM OCA 69 285

proteins of the plant ferredoxin type. The latter two proteins cannot accept electrons from P. oleovorans reductase and thus do not substitute for rubredoxin in the hydroxylation reaction. On the other hand, even though rubredoxins from the anaerobic bacteria P. elsdenii, D. gigas, and C. pasteurianum do accept electrons from the P. oleovorans reductase, they are still unable to substitute for P. oleovorans rubredoxin in the overall hydroxylation reaction. Taken together, these facts suggest the possibility that our rubredoxin does not simply play an electron transport role in this system, but that it participates in a much more direct way in the binding and activation of oxygen. This possibility is particularly intriguing when considered together with the fact that our rubredoxin contains an extra sulfhydryl group near each FeS ligation site, and such an extra sulfhydryl is not present in the anaerobic rubredoxins which are non-functional in the oxygenation reaction (See Below, Sections C and E).

Recognizing these unique features of the P. oleovorans system, we unequivocally established in our first two papers (references 46 and 47) that this system indeed carries out the epoxidation of olefins even more readily than the "normal" hydroxylation of alkanes, and does so with a variety of simple aliphatic olefin substrates. We showed that epoxidation exhibited all the expected enzymatic characteristics and stoichiometries, and competition studies suggested that a single epoxidase/hydroxylase carries out both reactions, although this important point remained uncertain. We therefore referred to this system as the P. oleovorans epoxidation/hydroxylation system (hereafter abbreviated POEHS) and began to carry out the detailed studies summarized in the following section of this proposal.

C. Summary of Progress to Date

In this section, progress to date is summarized very briefly. However, for each topic, references to our published papers are provided, where additional details can be found.

Since our initial discovery of the epoxidative activity of POEHS, our work has gone through essentially two phases and is now into a third phase. In Phase I we outlined the general characteristics and specificity of the epoxidation reaction, discovered and analyzed its stereoselectivity, and analyzed the reaction for mechanism by carrying out direct comparisons with chemical model systems and utilizing deuterated substrates. Because of the difficulties inherent in isolating two of the three protein components in pure form, we chose to carry out most of these studies using partially purified enzyme preparations. This allowed us to generate easily sufficient quantities of products for preparative isolation and examination by physical techniques (e.g. partially relaxed Fourier Transform nmr or nmr with shift reagents). We were fortunate in these studies because the epoxidation reaction we had discovered is essentially a metabolic dead end; i.e. products accumulate and are slowly metabolized, if at all. The following are our MAJOR conclusions from this phase:

-- The substrate specificity of POEHS is far different than that predicted on the basis of chemical reactivity patterns observed for non-enzymatic agents. Together with complementary inhibitor binding studies this demonstrates the importance of hydrophobic binding factors related to the alkyl chain configuration, and led us to rely on stereochemical analyses and configurational studies -- not specificity -- for mechanistic conclusions. (Details in references 46, 47, 48, 49, and our review reference 2).

-- Stereochemical analyses of epoxide products demonstrated a very high degree of

stereoselectivity in epoxidation (e.g. >90% of the epoxide formed from octadiene is the R(+) enatiomer). Our stereochemical results require that oxygen attack at C-2 occurs exclusively from one face of the olefin (e.g. the si-si face of octadiene). (Details in references 56 and 57).

-- Through use of the specifically synthesized substrate trans,trans -1,8-dideutereo-1,7-octadiene on a preparative scale, and isolation of its epoxide product followed by configurational analysis using partially relaxed FT NMR, we established that enzymatic epoxidation does not proceed with retention of the original olefin geometry . These experiments were designed as a direct test of the "oxenoid" mechanism (63), since olefin epoxidation by peracids is among the most well studied of all oxenoid reactions in organic chemistry (64-69), and these exhibit absolute syn steroespecificity (65-69). As Berti has pointed out (66), among the very large number of epoxidations of known steric course, there has not been reported a single instance of cis olefin giving threo or trans olefin giving erythro epoxide. Similarly, among the oxo transition metal complexes, which have been considered as possible models for oxygenase reactions (70), chromyl acetate (71) and molybdenum oxo complexes (72) are known to epoxidize olefins with retention of geometry, and the same is true of transition-metal catalyzed epoxidations by alkyl hydroperoxides (73,74). Taken together, our stereochemical and configurational results are inconsistent with an "oxenoid" mechanism and we have evaluated possible two step mechanisms involving radical and/or carbonium ion intermediates in reference 58. (Details in references 58, 59, 60, 61, 50). An interesting perspective is that our mechanistic arguments were presented and published (58, 59) at a time when they went "against the grain" of the prevalent tacit assumption that all oxygenase reactions were concerted. However, beginning with the work of Groves (75, 76) whose conclusions were strikingly similar to ours, and continuing to current work of others (see, for example, reference 77), the notion of radical and/or carbonium ion intermediates in P:450 oxygenases has gained much support.

After publication of these basic findings, we made the decision to temporarily set aside mechanistic studies of epoxidation . Thus, we initiated phase II of our program, in which we shifted our attention to the individual enzyme components, and the following are our MAJOR accomplishments in this phase:

-- The "epoxidase" has finally been purified and stabilized through the procedure of removing the essential iron atom and and purifying the apoenzyme, which is quite stable to prolonged storage. Iron is then replaced when the protein is assayed. This ends a major operational problem which has been intractable over many years, and establishes the identity of the "epoxidase" and "w-hydrolase" (Details in 50, 51, and 52).

-- Rubredoxin has been immobilized and shown to exhibit normal spectral properties and redox potential and to be catalytically competent in the immobilized state , thus making possible a number of affinity chromatography experiments to probe for interactions between the protein components (Details in reference 53).

-- We have replaced the native Fe atoms of rubredoxin with cobalt, to give Co(II)-rubredoxin, with both metal binding sites occupied (2 atoms of Co(II) per rubredoxin molecule). This represented the first example of the chemical substitution of a metal in an iron sulfur protein. Cobalt rubredoxin was characterized as to metal content, and its spectral properties are fully consistent with the presence of two Co(II) atoms in rubredoxin-type binding sites, exhibiting d-d transitions and charge transfer bands of the intensity and position predicted from the data with model compounds. The CD, MCD and resonance Raman spectra were also compared for both native and cobalt rubredoxin. Strikingly, cobalt rubredoxin

is much more stable than the native enzyme toward denaturants and metal dissociation, and no evidence for nonequivalence of the two cobalt binding sites was obtained. Cobalt rubedoxin also retains its ability to complex with reductase (Details in references 54, 55, 51, and 52).

From our point of view, the preparation of cobalt rubredoxin is especially significant for the following reason. P. oleovorans rubredoxin differs from those of the anaerobes in that an extra thiol group is located near each metal binding site. It has thus been an important goal to probe for two "reactive," non-metal-protected sulfhydryls using selective chemical modification, in order to test for a possible role in electron transport or catalysis. To date, this has been impossible due to the lability of the second iron in (2 Fe) rubredoxin, which precludes selective modification of two sulfhydryls per protein molecule. In sharp contrast, with (2 Co)-rubredoxin we have been successful in demonstrating the presence of two highly reactive sulfhydryl groups, and have prepared cobalt rubredoxin with only these sulfhydryls modified, on a preparative scale. Thus, a number of experiments with S-modified cobalt or iron rubredoxin become possible, as described in Sections IID and IIE.

Finally, the much enhanced stability of cobalt rubredoxin toward both denaturation and metal dissociation should make this species ideal for affinity chromatography studies with both epoxidase and reductase using immobilized cobalt rubredoxin.

Within the last year or so, we have initiated Phase III of our program in which we have resumed mechanistically- oriented studies with purified enzyme components (SEE BELOW).

D. Specific Objectives of Proposed Work

As set forth in Section IIA (page 3), we are proposing to continue our analysis of the oxygenation reactions carried out by the POEHS, which is a representative of a highly important but poorly understood class of monooxygenase involving an essential "non-heme" iron atom.

We have now entered the third phase of our program. In Phase I , we outlined the general reaction characteristics, specificity, and regio- and stereoselectivity of the epoxidation reaction, and we probed for mechanism by combining stereochemical data with our results from configurational FT nmr studies with specifically deuterated substrate. In Phase II , we shifted our attention away from studies of the epoxidation reaction to studies on the individual enzyme components of the epoxidation system. In this second phase we succeeded in obtaining evidence for the identity of the "epoxidase" and "hydroxylase," preparing and carefully characterizing immobilized, cobalt-substituted and specifically thiol-modified rubredoxin, and also stabilizing the reductase. As a result, procedures for obtaining and maintaining the stability of all of the enzyme components are in hand. We have also learned how to manipulate the rubredoxin molecule considerably. Finally, in the initiation of Phase III within the last year, we have confirmed our findings that epoxidation proceeds with loss of olefin configuration using purified enzyme preparations (see page), we have made the important discovery of aldehyde formation via intramolecular rearrangement during epoxidation (see page 9), and have initiated a series of experiments designed to settle the intriguing question of whether the "extra" thiols of rubredoxin play a functional role (see page 14).

ANNUAL TECHNICAL LETTER

NSF Grant PCM79-18334

December 15, 1979 - December 31, 1980

Enzymatic Epoxidation and Oxygen Activation

Submitted By

Sheldon W. May

Principal Investigator

School of Chemistry

Georgia Institute of Technology

Atlanta, Georgia 30332

I. PUBLICATIONS AND PRESENTATIONS SINCE START OF PROJECT

The following is a list of our publications and presentations since the last report. Appropriate copies of abstract, preprints and reprints have been sent to NSF as they have become available.

"Separation Techniques Based on Biological Specificity", S. W. May and L. M. Landgraff, Separat. Science, 5, 227 (1979).

"Enzymatic Epoxidation", S. W. May, Enz. Microb. Technology, 1, 15 (1979).

"Enzymatic Production of Saturated Ketones from Allylic Alcohols", S. W. May, M. S. Steltenkamp, K. R. Borah, A. G. Katopodis and J. R. Thowsen, J. Chem. Soc. Chem. Commun., 845 (1979).

"Enzymatic Oxygenation of Hydrocarbons", R. S. Phillips and S. W. May, Symposium Series, ACS, 24, 851 (1979).

"Asymmetric Sulfoxidation by Dopamine-β-Hydroxylase, An Oxygenase Heretofore Considered Specific for Methylene Hydroxylation", S. W. May and R. S. Phillips, J. Am. Chem. Soc., 102, (1980).

"Enzymatic Sulfur Oxygenation Reactions", S.W. May and R. S. Phillips, Enzym. Microb. Technology, 2, 490 (1980).

"Dopamine-β-Hydroxylase: Demonstration of Enzymatic Ketonization of the Product Enantiomer", S. W. May, P. W. Mueller, R. S. Phillips and H. H. Herman, J. Biological Chem., 255, 2258 (1981).

Enzymatic Production of Ketones and Purification of a Secondary Alcohol Dehydrogenase", S. W. May, K. R. Borah, M. S. Steltenkamp and J. R. Thowsen, Fed. Proceed, 39, 1300 Abs. (1980).

II. SUMMARY OF RESEARCH PROGRESS

Effects of Metal Substitution and Chemical Modification on the Properties of Rubredoxin - In this phase of our program our objectives were to utilize cobalt rubredoxin to probe for a possible reductant/effector role for rubredoxin in order to clarify questions which have been raised by previous work regarding the precise catalytic role of this protein. We also wished to make use of our ability to chemically modify specifically the two "extra" thiol groups in rubredoxin in order to demonstrate once and for all whether they play a functional role in the catalytic activity of this protein. Finally, we wished to attempt to prepare hybrid Fe/Co rubredoxins.

To date, we have successfully accomplished all of these initial objectives. Our work with cobalt rubredoxin has conclusively established that this protein indeed plays an effector role in addition to its function in transferring electrons to the epoxidase. In cobalt rubredoxin, the effector function remains while electron transfer is precluded due to the potential of the cobalt atom. Thus, molecular-level information regarding the precise role of the various protein components is beginning to emerge, similar to that which has been obtained with the much more extensively studied P-450 systems. We have succeeded in cleanly modifying the two "extra" thiol groups of rubredoxin which are absent in the rubredoxins from the anaerobic bacteria. Our specifically modified rubredoxin was carefully and exhaustively characterized using amino acid analyses, radioactive labelling, specific CNBr cleavage to demonstrate the location of the modified sulfhydryls, and spectral analysis. Once modified, the cobalt atoms were removed from the rubredoxin, iron put back in, and the resultant modified rubredoxin species examined for catalytic activity. We have now found that sulfhydryl modification does not abolish the ability of rubredoxin to function in the epoxidation system, thus establishing once and for all that the extra sulfhydryls *do not play a direct functional role*. Finally, we have succeeded in preparing and characterizing hybrid Fe/Co rubredoxins much more successfully than we anticipated at the start of these studies. The properties of the hybrid rubredoxins have started to provide insight into the three dimensional structure of the protein and the molecular basis for the non-equivalence of the metal binding sites at the two terminii.

In experiments which we did not forsee at the time that our most recent proposal was written, we are collaborating with Dr. James Anderson of the University of Georgia in determining reduction potentials for the various metal-substituted rubredoxins using direct potentiometric techniques. To date, we have obtained a provisional value of 100+ 50mv for cobalt rubredoxin. This value not only explains the ability of cobalt rubredoxin to reduce cytochrome c, but also provides an initial estimate of the potential of the epoxidase, since cobalt rubredoxin reduces cytochrome c but cannot transfer electrons during the oxygenation process. We estimate that the potential of epoxidase is in the 0 mv range, which is highly consistent with potentials reported for heme-containing monooxygenases.

Stabilization and Characterization of the Epoxidase - Recently, we have made a break through in improving our isolation procedure for the epoxidase, and also for the related secondary alcohol dehydrogenase (SADH) which we have been studying recently. Our new procedures allow complete solubilization of these proteins and are providing preparations amenable for the spectral and Raman studies described in the proposal. We expect to be heavily involved in such work during the next year of this research project.

Emerging Studies with the Copper Monooxygenase, Dopamine-β-Hydroxylase - Our interest in metal substitutions with the P. oleovorans epoxidation system, and the stereochemical studies with this system in which we have been involved over the past several years, has lead us to embark on an additional direction which we did not at all forsee at the initiation of this phase of our program. However, work in this direction is beginning to provide what we regard as some of our most promising and exciting results. It is our hope that these studies will provide direct comparisons between mamalian and bacterial monooxygenases and establish a mechanistic unity among oxygenases utilizing different metal atoms directly in catalysis. To date, our work with Dopamine-β-Hydroxylase has established two new monooxygenase activities for this enzyme -- stereospecific sulfoxidation of sulfides and ketonization of enantiomers of the normal hydroxylation products. We have now successfully carried out a systematic study of both the effects of substrate secondary structure and of various substituents on the sulfoxidation and hydroxylation reactions. Our results have allowed us to make a direct mechanistic comparison between Dopamine-β-Hydroxylase, cytochrome P-450 and chemical model systems, and have allowed us to suggest a unified mechanism for Dopamine-β-Hydroxylase catalyzed oxygenation reactions. These results are described more fully in the reprints and pre-prints attached to this report. We are confident that this new exciting direction in our research will compliment our continuing studies with the P oleovorans epoxidation/hydroxylation system and provide a unified view of enzymatic oxygen activation.

6-33-655

ANNUAL TECHNICAL LETTER

NSF Grant PCM79-18334

January 1, 1981 - December 31, 1981

Enzymatic Epoxidation and Oxygen Activation

Submitted By

Sheldon W. May

Principal Investigator

School of Chemistry

Georgia Institute of Technology

Atlanta, Georgia 30332

1. PUBLICATIONS AND PRESENTATIONS SINCE START OF PROJECT

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"Dopamine-β-Hydroxylase: Comparative Specificities and Mechanisms of the Oxygenation Reactions," S.W. May, R.S. Phillips, P.W. Mueller and H.H. Herman, J. Biol. Chem., 256, 8470 (1981).

"Dopamine-β-Hydroxylase: Novel Synthetic Substrates and Inhibitors," R.S. Phillips, H.H. Herman, P.W. Mueller and S.W. May, Fed. Proceed, 40, 1670 (1981).

"Enzymatic Catalysts for the Stereo and REgioselective Oxyfunctionalization of Organic Substrates," S.W. May, Enzyme Engineering, 6, In Press (1982).

"Bioactivation of Catha edulis Alkaloids: Enzymatic Ketonization of Norpseudoephedrine," S.W. May, R.S. Phillips, H.H. Herman and P.W. Mueller, Biochem. Biophys. Res. Comm., 104, 38 (1982).

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In our last progress report, we reported that we had succeeded in cleanly modifying the two "extra" thiol groups of rubredoxin which are absent in the rubredoxins from the anaerobic bacteria. Our specifically modified rubredoxin has now been carefully and exhaustively characterized using amino acid analyses, radioactive labeling, specific CNBr cleavage to demonstrate the location of the modified sulfhydryls, and spectral analysis. Once modified, the cobalt atoms have now been successfully removed, iron put back in, and the resultant modified rubredoxin species examined for activity in supporting epoxidation. We find that sulfhydryl modification greatly reduces the ability of rubredoxin to function in the epoxidation system, thus indiciating that the extra *sulfhydryls play an important functional role.* In related work, we have now succeeded in preparing and characterizing hybrid Fe/Co rubredoxins and during the past year we have studied these hybrid species extensively. Intriguingly, stoichiometric activity titrations indicate that while maximal activity is obtained with a 2:1 ratio of native rubredoxin to epoxidase, Fe/Co hybrid rubredoxins exhibit maximal activity at a 1:1 ratio of these proteins. Our current thinking is that this difference is related to a possible regulatory "effector" role for rubredoxin, not unlike the effector role postulated for putidaredoxin in P-450 oxygenase systems.

Stabilization and Purification of the Epoxidase - A major breakthrough has occured in our laboratory in that the "epoxidase" has finally been purified and stabilized, thus ending a major operational problem which has remained intractable for many years. Our procedure involves removing the essential iron atom and purifying the apoezyme, which is quite stable to prolonged storage. Iron is then replaced when the protein is dissolved in the normal assay mixture. Thus, we have finally been able to define the specificity and spectral properties of the purified epoxidase. We can now say with certainty that the spectrum of the epoxidase does not exhibit a charge transfer band in the 400 mn region which would provide a handle for studying this protein using resonance Raman spectroscopy..

Mechanistic Studies - As is set forth in detail in the body of our NSF proposal, our previous stereochemical and mechanistic studies with deuterated substrates have provided highly significant results. For example, in 1977 we published the first evidence for a radical mechanism in an oxygenase reaction, a conclusion which has since been duplicated by several other investigators in other systems. Our earlier work was of necessity carried out with crude enzyme preparations, since it was impossible to purify and stabilize the epoxidase. With pure epoxidase now in hand, we have repeated the key mechnistic experiment -- testing for retention of configuration in a specifically deuterated olefin substrate. Our results again demonstrate loss of olefin configuration, confirming our earlier conclusions. A communication reporting these findings is currently in preparation.

Studies with Dopamine-β-Hydroxylase - Our interest in metal substitution with

P. oleovorans epoxidation system, and the stereochemical studies with this system in which we have been involved over the past several years, has lead us to embark on an additional direction wihch we did not at all forsee at the initiation of this phase of our program. However, work in this direction is beginning to provide what we regard as some of our most promising and exciting results. It is our hope that these studies will provide direct comparisons between mammalian and bacterial monooxygenases and establish a mechanistic unity among oxygenases utilizing different metal atoms directly in catalysis. To date, our work with Dopamine-β-Hydroxylase has established two new monooxygenase activities for this enzyme -- stereospecific sulfoxidation of sulfides and ketonization of enantiomers of the normal hydroxylation product. We have now successfully carried out a systematic study of both the effects of substrate secondary structure and of various substituents on the sulfoxidation and hydroxylation reactions. Our results have allowed us to make a direct mechanistic comparison between Dopamine-β-Hydroxylase, cytochrome P-450 and chemical model systems, and have allowed us to suggest a unified mechanism for Dopamine-β-Hydroxylase catalyzed oxigenation reactions. These results are described more fully in the reprints attached to this report. We are confident that this new exciting direction in our research will compliment our continuing studies with the P. oeeovorans epoxidation/hydorxylation system and provide a unified view of enzymatic oxygen activation.

NATIONAL SCIENCE FOUNDATION
Washington, D.C. 20550

FINAL PROJECT REPORT
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PART I–PROJECT IDENTIFICATION INFORMATION

1. Institution and Address	2. NSF Program	3. NSF Award Number
Georgia Tech Research Institute Georgia Institute of Technology Atlanta, GA 30332		PCM-7918334
	4. Award Period From 12/15/79 To 11/30/83	5. Cumulative Award Amount $70,000

6. Project Title

Enzymatic Epoxidation and Oxygen Activation

PART II–SUMMARY OF COMPLETED PROJECT *(FOR PUBLIC USE)*

The significance of oxygenases--enzymes which catalyze the direct insertion of molecular oxygen into organic substrates--to biochemistry, medicine, and considerations of public health has by now been very well established. While P-450-and flavin-containing oxygenases have been intensively studied on a molecular level, the state of our understanding of non-heme iron oxygenase catalysis, particularly the monooxygenases of this class, is by comparison, poor indeed. The broad objective of this project was to continue our analysis of the oxygenation reactions carried out by the non-heme-iron-containing monooxygenase, the epoxidation/hydroxylation system of P. oleovorans. Our work involved mechanistic studies, the design and evaluation of novel substrate analogs and suicide substrates, metal replacement and other active-site-directed chemical techniques, and finally, initial attempts to apply physical techniques to non-heme iron monooxygenases. Our work represents one of the very few efforts to define the molecular details of non-heme iron monooxygenase catalysis.

PART III–TECHNICAL INFORMATION *(FOR PROGRAM MANAGEMENT USES)*

1. ITEM *(Check appropriate blocks)*	NONE	ATTACHED	PREVIOUSLY FURNISHED	TO BE FURNISHED SEPARATELY TO PROGRAM	
				Check (✓)	Approx. Date
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d. Information on Inventions	✓				
e. Technical Description of Project and Results			✓		
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2. Principal Investigator/Project Director Name *(Typed)*	3. Principal Investigator/Project Director Signature	4. Date
✓ Sheldon W. May		2/15/85

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